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Calmodulin Antagonists as Potential Antifungal Agents

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The ubiquitous proteins, the calmodulins (CAMs), play a key role in cellular proliferation.[1] They are highly conserved in most higher eukaryotes, but there is significant divergence in fungal CAMs, compared with those from other species, raising the possibility that these might be targets for novel antimycotic drugs.[2]

There are a few reports of the inhibitory effects of antifungal agents on calmodulin-mediated systems. It has been shown[3] that chloraniformethan inhibits CAM-dependent cyclic nucleotide phosphodiesterase (PDE), and the clinically useful azole antifungal ketoconazole at low micromolar concentrations inhibits this enzyme[4,5] and also CAM-dependent nitric oxide synthase.[6] To our knowledge, however, there have been no attempts to develop calmodulin antagonists specifically as antifungal agents.

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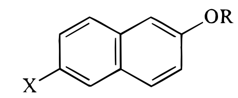
As lead compounds we used the naphthalenesulfonamides, introduced by Hidaka and Tamaka[7] and further developed by MacNeil *et al.*[8] They have the general formula $ArSO_2NH(CH_2)_nNH_2$, where Ar is a 1- or 2-naphthyl residue, preferably halogenated and *n* lies between 6 and 10 for maximum inhibitory potency. Examples are W7 and J8 (Fig. 1). In our initial attempts to modify these compounds, the sulfonamide linkage in W7 was replaced by polar amide, urea, or thiourea groups. In each case the ability of the resulting compounds to inhibit CAM-dependent PDE was much reduced. However, substitution of the SO_2NH moiety by a *non-polar* ether or thio-ether linkage gave compounds equipotent with corresponding sulfonamides, and the ether group was adopted in all subsequent compounds synthesised.

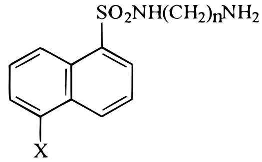
Since W7 inhibits calcium-activated transglutaminases at concentrations similar to those required to bring about calmodulin antagonism,[9] the terminal side-chain primary amine necessary for this competing inhibition was replaced in our compounds by a number of tertiary amines. The most effective of these were found to be derived from pyrrolidine or piperidine, presumably because the pKa of *N*-alkylpyrrolidines (10·32) and piperidines (10·1) is close to that of primary alkylamines (10·64), whereas incorporation of imidazole (pKa 7·33) or morpholine (pKa 7·41) gave poorer antagonists. A similar substitution of pyrrolidine for the primary amino group has recently been found to enhance the CAM-inhibitory properties of some tamoxifen derivatives.[10]

In agreement with the earlier studies,[7,8] the potency as PDE inhibitors of our compounds, now with the general structure $Ar–O–(CH_2)_n–NR_2$, increased with the length of the alkyl side-chain. There was a marked rise at $n = 4$ to 6, with a further slight improvement up to $n = 9$. Although, in most of the compounds prepared, the aryl group was 1- or 2-naphthyl, 4-benzothiophenyl or 2-dibenzofuranyl residues were also effective. Interestingly, benzofuranyl-containing compounds were significantly less potent than analogous benzothiophenes. Representative results are shown in Table 1. The inhibition of CAM-dependent PDE was measured by the modified method of Thompson *et al.*[11] in which tritiated AMP liberated from cAMP was converted by snake venom to adenosine. The synthesis of the inhibitors, by standard methods, is described in a patent.[12]

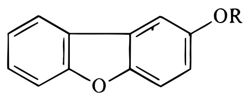
The test organism selected for assaying antifungal activity in our compounds was *Pythium ultimum* Trow.



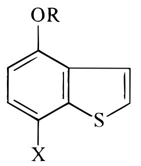
1 X = H, R = $(CH_2)_4$-1-piperidinyl
2 X = Br, R = $(CH_2)_4$-1-piperidinyl
3 X = Br, R = $(CH_2)_2$-1-pyrrolidinyl
4 X = Br, R = $(CH_2)_4$-1-pyrrolidinyl
5 X = Br, R = $(CH_2)_6$-1-pyrrolidinyl
6 X = Br, R = $(CH_2)_8$-1-pyrrolidinyl
7 X = I, R = $(CH_2)_6$-1-pyrrolidinyl
8 X = I, R = $(CH_2)_8$-1-pyrrolidinyl
9 X = Br, R = $(CH_2)_4SO(CH_2)_3$-1-pyrrolidinyl
10 X = Br, R = $(CH_2)_6SO(CH_2)_3$-1-pyrrolidinyl



W7 X = Cl, n = 6
J8 X = I, n = 8



11 R = $(CH_2)_4$-1-piperidinyl
12 R = $(CH_2)_4$-1-pyrrolidinyl
13 R = $(CH_2)_3SO(CH_2)_2$-1-pyrrolidinyl



14 X = H, R = $(CH_2)_4$-1-piperidinyl
15 X = H, R = $(CH_2)_6$-1-pyrrolidinyl
16 X = Br, R = $(CH_2)_6$-1-pyrrolidinyl

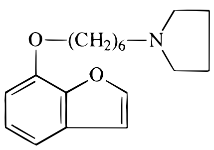


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Fig. 1. Structures of compounds discussed.

This fungus is an easily cultured plant pathogen which, as an oomycete, does not synthesis ergosterol and is unaffected by fungicides which inhibit sterol biosynthesis.[13]

The *Pythium* was cultured on potato dextrose agar (PDA) and incubated at 25°C. Test compounds, dissolved in aqueous DMSO, were mixed at known concentration into the PDA in duplicate Petri dishes. Three plugs of the *Pythium* were introduced into each dish, and radial development of the colony was measured at 24 and 48 h. Percentage inhibition, defined as $100 - 100 \times$ (growth with test compound/growth of control) plotted against concentration of test compound gave the IC_{50} values shown in Table 2. Although approximate, the figures allow a relative ranking into potent and less potent inhibitors.

In general, inhibition of the growth of *Pythium* paralleled calmodulin antagonism. Thus, within an homologous series (e.g. compounds **3**–**6**) potency increased with chain length. With compound **15**, however, which was very active in the PDE assay, inhibition of fungal

TABLE 1
Inhibition of PDE by Compounds

Compound	IC_{50} (μM)
1	56(±6)
2	9(±2)
11	20(±3)
14	1·8(±0·1)
15	0·22(±0·04)
17	204(±13)

TABLE 2
Effect of Selected Compounds on Growth of *Pythium*

Compound	IC_{50} (μM)
3	163(±24)
4	33(±6)
5	32(±14)
6	29(±14)
7	91(±24)
8	43(±10)
9	41(±14)
10	21(±2)
13	41(±10)
15	137(±14)
16	58(±15)

growth fell off rapidly with dilution, in a manner which was not dose-dependent. This was attributed to metabolism of the antagonist, possibly by hydroxylation *para* to the ether linkage, or by oxidation of the thiophene sulfur atom. Experiments to identify metabolites are in progress. Incorporation of a bromine atom in an appropriate position (compound **16**) gave an analogue with reasonable potency, and fungal inhibition proportional to concentration.

In the development of J8, an iodine substituent was found[8] to be a more effective enhancer of calmodulin antagonism than bromine or chlorine. We were therefore surprised to note that iodo-compounds **7** and **8** were less active against *Pythium* than the corresponding bromo-compounds **5** and **6**.

The best inhibitors, containing both bromonaphthyl and long alkyl chains, are extremely insoluble in aqueous media, but the solubility of the compounds could be markedly improved by incorporation of sulfoxide groups into the side chain (compounds **9**, **10**, **13**) with only a marginal loss in activity.

The development of our series of calmodulin antagonists as potential anti-fungal agents was carried out after our preliminary work with the PDE assay, which was no longer available to us. To test that the compounds developed later were still active as CAM inhibitors a number were assayed[14] in a CAM-dependent myosin light chain kinase (MLCK) screen, and showed IC_{50} values equal to or better than those of W7 and J8 (Table 3).

TABLE 3
Inhibition of CAM-dependent MLCK by Compounds

Compound	IC_{50} (μM)
W7	48
J8	26
6	10·7
9	34
16	25

In summary, a number of novel calmodulin antagonists have been synthesized, and their inhibitory action on the growth of *Pythium* appears to correlate with their potency as calmodulin antagonists, though it has not been proved that this is their mode of antifungal action.

Further studies are in progress to assess the inhibitory activity of the compounds against a variety of higher fungi.

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